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BREX is a novel phage resistance system widespread in microbial genomes

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

Editor: David del Alamo

1st Editorial Decision 22 July 2014

Thank you for the submission of your manuscript entitled "BREX, a phage resistance system widespread in microbial genomes" and for your patience while it has been evaluated. We have now received the full set of reports from the referees, which I copy below.

As you can see from their comments, we have 2 rather positive and 2 rather negative referees. While referees #2 and #3 agree on the high and general interest of your work, referees #1 and #4 believe that a much deeper functional characterization of the BREX systems would be required before your manuscript can be published in The EMBO Journal. Considering their comments and the already rather large amount of data provided together with the highly valuable meta-genomic analysis you perform, we believe that further functional characterization, although obviously interesting, would be out of the scope of the present manuscript (most likely of any single manuscript indeed). Naturally, any further addition of data in this direction would only add to the interest of your work but we do not deem this fundamental for the acceptance of your manuscript.

That being said, referee #4 expresses some concerns regarding the experimental evidence that is actually presented in the manuscript, and these points, particularly number 7, 8, 9, 10, 11, 17 and 18,

will need to be addressed, experimentally if required. In addition, referee #1 suggests a relatively simple but highly informative experiment in its point 1 that we believe constitutes a reasonable addition to your paper. Other minor points from these or referee #2 should be addressed in your point-by-point response.

Although I believe the issues to be dealt with are relatively straightforward, please do not hesitate to contact me if you have any questions, need further input on the referee comments or you anticipate any problems along the revision process.

In summary, given these evaluations, I would like to invite you to submit a revised version of the manuscript. It is 'The EMBO Journal' policy to allow a single round of major revision only, which should be submitted within the next three months. Should you foresee a problem in meeting the three-month deadline, please let us know in advance and we may be able to grant an extension.

I would also like to point out that as a matter of policy, competing manuscripts published during this period will not be taken into consideration in our assessment of the novelty presented by your study ("scooping" protection). However, we would appreciate if you contact me as soon as possible upon publication of any related work in order to discuss how to proceed.

When preparing your letter of response to the referees' comments, bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process initiative, please visit our website: http://emboj.msubmit.net/html/emboj_author_instructions.html#a2.12

Thank you very much again for the opportunity to consider your work for publication. I look forward to your revision.

REFEREE REPORTS:

Referee #1:

In this manuscript data are presented that a wide-spread bacterial gene cluster centered on a pglZ-like gene from B. sereus can provide resistance to a variety of phages when expressed in a heterologous B. subtilis host. Some preliminary data on the function of this phage exclusion system are presented that suggest that the new system (named BREX) may be distinct from the Pgl exclusion system discovered in the early eighties by a Russian group (a conclusion that shall be regarded as tentative as the Pgl phenotype itself is mechanistically not defined); that BREX does not prevent phage absorption/DNA entry but does prevent phage DNA replication. Finally, a BREX cluster DNA methylase is shown to be essential for phage protection, while its activity is shown to be directed to the surrogate bacterial host, not phage DNA. These are all interesting observation that might point to a new defence system which may (or may not) acquire the fame of CRISPR-Cas in the future. However, the study appears to be too preliminary with now mechanism - not even a hypothesis - of BREX function shown/proposed. In the absence of this crucial evidence, the paper probably belongs to a specialised microbiology journal.

Referee #2:

In this well written manuscript, the authors describe a novel microbial defense system with properties distinct from traditional restriction-modification (R-M) and other known mechanisms, such as CRISPR-Cas and abortive infection (Abi). The six-gene cluster found in Bacillus cereus, as the most abundant subtype of the BREX system, successfully recapitulated phage resistance. Interestingly, it does not fully protect against all phages tested, implying a possible evolution of

counter measures for some bacteriophage. The authors explore further details, confirming that BREX (1) allows phage adsorption but prevents subsequent phage DNA replication. While BREX (1) methylates bacterial DNA, it does not degrade unlabeled phage DNA unlike most R-M systems, does not cause toxicity or growth deficiencies when the methylation gene, pglX, is knocked out, and it does not promote cellular suicide upon phage infection, making it distinct from abortive infection. The authors' extensive analysis of 1500 bacterial and archaeal genomes revealed at least five additional subtypes (2-6), suggesting that the BREX defense systems are widespread and define a superfamily of phage resistance that undergo extensive horizontal gene transfer.

There are essentially no major flaws in this well-written paper, which represents a significant contribution in the discovery of a novel phage defense system that is abundant in bacteria and archaea. A few minor problems could be easily corrected:

Page 7, last paragraph: "The Pgl phenotype observed in S. coelicolor A3 predicts that the first infection cycle by the phage [would] be successful, producing viable phage progeny."
Page 27, Fig. 2: graph C: a unit such as (-fold) should be included on the Y axis; graph D: the labels "Phage" and "Non-lysogen" for rows on the gel should be switched; legend: add U, uninfected
Page 28, Fig. 3: graph C is unlabeled and a unit such as (-fold) should be included on the Y axis.
Page 29, Fig. 4, legend: "Curves depict culture dynamics of strains [lacking] BREX..."
Page 37, Fig. S2, legend: "Presented are dynamics for all tested phages except for those already presented in Figure [1]. Axes are as in Figure [1]."

Referee #3:

Goldfarb and Sberro et al. describe a novel super-family of Bacteriophage Exclusion (BREX) systems in bacteria and archaea. Although these innate defense systems also include a previously characterized anti-phage system from Streptomyces (PglZWXY; Chivenova 1982; Sumby & Smith 2002), the current study is a major extension that reveals a the broad distribution of BREX in about 10% of the available bacterial and archaeal genomes, and the existence of six BREX sub-types. More specifically, thorough experimental analysis is described of the 6-component system of Bacillus cereus, by its functional expression in B. subtilis. The cluster of 6 genes encode: a NusB-like RNA-binding protein (BrxA), an unknown small protein (BrxB), a P-loop ATPase (PglY/BrxC), a methyl transferease (PlgX), an alkaline phosphatase (PlgZ), and a Lon-like protease (BrxL). It is demonstrated that the heterologous BREX system provides B. subtilis with significant protection from a set of lytic and lysogenic phages. The fact that the system does not protect against all tested phages is explained by assuming the existence of counteracting viral anti-BREX systems. In a series of experiments, the authors gain some first insight in the BREX mechanism. Unlike earlier reports on the Streptomyces Pgl-system, the BREX system prevents the production of phage upon the first infection, and no integration of phages is observed. BRX is not an abortive infection system, and it does not block phage adsorption. However, the fact that phage DNA levels do not increase upon infection, indicate that phage replication is significantly inhibited by the BREX system. Sequence analysis revealed that in the presence of BREX, the host DNA (but not the phage DNA) was methylated at TAGGAG motifs. Unlike classical Restriction/Modification systems, the phage DNA is not being degraded. Based on the in silico and experimental analyses the authors draw some conclusions on the functionality of the system and its components. This study provides a strong basis for future unravelling of the molecular mechanism of this intriguing defense system.

Major concern

1. The authors admit that many of the mechanistic details of the BREX system remain to be elucidated. Obviously, it is not difficult to propose some additional experiments. However, given the large set of data provided, it seems fair to wait for a follow-up study that addresses these matters. One easy experiment could be implemented. The authors should compare plasmid transformation efficiencies of the B.subtilis strain with/without BREX, using a Bacillus-E.coli shuttle vector

(produced in E.coli). This will reveal whether BREX is a dedicated anti-virus system, or rather a general anti-invader system.

2. (p.18, line 1) - It is concluded that the function of the protease in type-1 can be replaced by a helicase in other subtypes. As these are completely different enzymatic activities, with distinct targets (protein vs. nucleic acid), the authors should elaborate on this.

3. The authors should indicate if TAGGAG motifs are randomly distributed, or if they mainly occur in 5'UTRs as part of RBS. If there is a bias, the authors should discuss the potential implications.

Minor concern

4. (p.6, line 9) - "these phages" > change to: "the former 7 phages"

5. (p.14, line 10 / p.18, line 23) - "co-horizontally transformed" > change to: "co-transformed horizontally".

Referee #4:

This paper addresses a novel phage defense system from Bacillus cereus that the authors have termed BREX (Bacteriophage exclusion). They show that transfer of a 6 gene cluster to B. subtilis confers resistance to a subset of Bacillus phages that infect the parental strain. The authors also claim that while a phage affected by BREX can adsorb to the hosts, DNA replication is prevented and lysogens are not established. Some of the BREX encoded genes are homologs of the unusual PGL system from Streptomyces coelicolor. Unlike PGL the phage infection in a BREX plus strain is prevented in the first round of infection so the authors claim the mechanism is different. As suggested by the presence of a putative DNA methyltransferase (PglX) the authors found that the DNA from the host containing BREX is methylated at a non-palindromic site, TAGGAG and that this methylation, and phage resistance, is dependent on the presence of the pglX homolog. During infection however there is no evidence for methylation of the phage genome. There was no evidence of phage DNA degradation by BREX-plus bacteria

The ms contains a large amount of bioinformatics analysis that indicates that BREX is a common gene cluster in bacteria and archaea.

Overall the ms is thin on experimental evidence for many of the claims. Only single experiments have been done to support claims such as the absence of abortive infection, the involvement of methylation in self/non-self recognition. Some of the claims are flawed. There is no overall model for the mechanism of BREX proposed.

Comments

1. Why did the authors chose to change the description of the resistance system, when a perfectly good precedent exists i.e. Phage Growth limitation. Moreover the term exclusion implies that phage are excluded from the host cells and the evidence is clearly not supporting this.

2. In many places in the ms the authors assume that the annotation is proof of activity. For example in the abstract lines 6 and 7 state that a lon-like protease, an alkaline phosphatase, a DNA methylase and an ATPase are involved in BREX but there is no validation for their biochemical activities or even whether the genes are required.

3. The role of the pglX homologue in BREX is demonstrated but not conclusively as it was not complemented. Given the instability of pglX homologues, it would be sensible to complement this mutation. None of the other genes thought to be required for BREX were shown to be required and yet all of the classification of the BREX systems depends on these homologues being active - in particular the pglZ homologues.

4. The assumption that phage that are resistant carry systems that evade BREX is wrong; these phages may simply not encode a target for BREX.

5. The typing of BREX systems is likely to breakdown as more combinations of genes close to pglY, (the single common homologue) are revealed. I don't think that the classification proposed would therefore be useful or sustainable. Any typing systems should be based on mechanism. The classification is flawed anyway as we don't know which genes are required.

6. P3. Paragraph 2, line 12. Insert 'motif' after 'methyltransferase'.

7. P6 paragraph 2 line 5. The growth kinetics are done under conditions in which the MOI is low so requiring many phage bursts before host lysis is observed. This should be made clear in the Results text.

8. The growth of the host strain does not appear to be optimal; as the data is not presented as a log OD600 against time, we cannot see if the bacteria were growing exponentially in the initial growth phase and there is a clear reduction in the doubling time at OD600nm of about 0.3 which is very low for Bacillus subtilis growing in LB. The phage amplification is not presented in these growth curves and I think this would be useful to get a complete view of the phage-host dynamics.

9. P7 paragraph 2, 4,5 and 6 lines up. This calculation is not clear and need further explanation/data to validate.

10. P8&9. A more detailed analysis over the initial time-points of growth post-infection need to be shown to demonstrate that there is no stalling of host cell division/ DNA replication. Note that the OD600 can continue to increase as cells filament if DNA replication is blocked. The claim that this system is not an abortive infection needs to be validated by viable counts of infected cells in BREX-plus cells.

11. P9. The use of Illumina reads to show the amplification of phage DNA compared to host DNA is not reliable as host DNA could be degraded. This might be already known for phi3T but it would not be known for phi3T infecting BREX plus cells. This experiment should be removed. The data using the southern blot is safer, but the readers need to see the amount of total DNA that was loaded onto the gel and blotted onto the membrane.

12. P11. Last line. Just because the so-called type I BREX systems are most common in the database, this does not mean it is most common in nature. Our view of what is common is skewed by what has been cultured and sequenced.

13. P12. Paragraph 3. This is pure speculation with no evidence to support the claim that the Pgl system requires two additional genes. In fact the evidence from Sumby and Smith suggests otherwise. Linkage does not prove any functional connection.

14. Pages 13 and 14 are descriptive only that lack any conclusions or biological insights.

15. P17. The authors show that replicating phages in a BREX plus strain are not methylated but how many target sequences do they have?

16. P18 lline 1&2. What kind of biochemical activity can be carried out by both a helicase and a protease? This is pure speculation. There is more speculation in the next paragraph.

17. Figure 1G. The growth kinetics of the phi105 infection is odd as both cultures BREX plus and BREX minus start to lyse at 6 hours. The pfu/ml over this time course would be informative. In fact the phi105 infections look more like the SPO1 infection of the BREX minus culture and suggests that BREX does not confer resistance to phi105 after all. The EoP of the phi105 cultures do not show plaques, but perhaps they are there but very small.

18. Figure 2B. The pfu/ml in the BREXplus culture with SPO1 decreases by about 1 log over 40 minutes an this is explained as adsorption, yet the pfu/ml in the BREX minus culture does not decrease - pfu/ml simply start to increase after about 30 minutes. Why this difference?

1st Revision - authors' response 23 September 2014

Editorial comments

…Considering their comments and the already rather large amount of data provided together with the highly valuable meta-genomic analysis you perform, we believe that further functional characterization, although obviously interesting, would be out of the scope of the present manuscript (most likely of any single manuscript indeed). Naturally, any further addition of data in this direction would only add to the interest of your work but we do not deem this fundamental for the acceptance of your manuscript.

Answer: Thank you!

That being said, referee #4 expresses some concerns regarding the experimental evidence that is actually presented in the manuscript, and these points, particularly number 7, 8, 9, 10, 11, 17 and 18, will need to be addressed, experimentally if required.

Answer: We provided detailed answers to all points by referee #4, including those indicated above, and changed the manuscript accordingly.

In addition, referee #1 suggests a relatively simple but highly informative experiment in its point 1 that we believe constitutes a reasonable addition to your paper.

Answer: We believe the editor meant point 1 of referee #3. We performed the experiments as suggested and added the results to the manuscript. We also included an additional supplementary figure (Fig S4) that presents the results of the suggested experiment.

Other minor points from these or referee #2 should be addressed in your point-by-point response.

Answer: We provided detailed answers to the minor points by referee #2.

Referee #1:

In this manuscript data are presented that a wide-spread bacterial gene cluster centered on a pglZ-like gene from B. sereus can provide resistance to a variety of phages when expressed in a heterologous B. subtilis host. Some preliminary data on the function of this phage exclusion system are presented that suggest that the new system (named BREX) may be distinct from the Pgl exclusion system discovered in the early eighties by a Russian group (a conclusion that shall be regarded as tentative as the Pgl phenotype itself is mechanistically not defined); that BREX does not prevent phage absorption/DNA entry but does prevent phage DNA replication. Finally, a BREX cluster DNA methylase is shown to be essential for phage protection, while its activity is shown to be directed to the surrogate bacterial host, not phage DNA. These are all interesting observation that might point to a new defence system which may (or may not) acquire the fame of CRISPR-Cas in the future. However, the study appears to be too preliminary with now mechanism - not even a hypothesis - of BREX function shown/proposed. In the absence of this crucial evidence, the paper probably belongs to a specialised microbiology journal.

Answer: In this manuscript we report on a novel set of defense systems that are widely spread among microbial genomes and provide broad defense against phages. We performed a thorough pan-genome analysis of this set of systems and their components, and provided a large amount of experimental data supportive of its defensive properties and characterizing some of its components. Admittedly we did not decipher the mechanism yet. However, as indicated by the editor, considering the already rather large amount of data provided together with the meta-genomic analysis, we believe that further functional characterization would be out of the scope of the present manuscript. Bacterial defense systems (e.g. CRISPR, R-M) have proven invaluable far beyond the microbiology community and we thus believe this multi-gene system may be of interest beyond a specialized microbiology journal.

Referee #2:

In this well written manuscript, the authors describe a novel microbial defense system with properties distinct from traditional restriction-modification (R-M) and other known mechanisms, such as CRISPR-Cas and abortive infection (Abi). The six-gene cluster found in Bacillus cereus, as the most abundant subtype of the BREX system, successfully recapitulated phage resistance. Interestingly, it does not fully protect against all phages tested, implying a possible evolution of counter measures for some bacteriophage. The authors explore further details, confirming that BREX (1) allows phage adsorption but prevents subsequent phage DNA replication. While BREX (1) methylates bacterial DNA, it does not degrade unlabeled phage DNA unlike most R-M systems, does not cause toxicity or growth deficiencies when the methylation gene, pglX, is knocked out, and it does not promote cellular suicide upon phage infection, making it distinct from abortive infection. The authors' extensive analysis of ~1500 bacterial and archaeal genomes

revealed at least five additional subtypes (2-6), suggesting that the BREX defense systems are widespread and define a superfamily of phage resistance that undergo extensive horizontal gene transfer.

There are essentially no major flaws in this well-written paper, which represents a significant contribution in the discovery of a novel phage defense system that is abundant in bacteria and archaea.

Answer: Thank you!

A few minor problems could be easily corrected:

Page 7, last paragraph: "The Pgl phenotype observed in S. coelicolor A3 predicts that the first infection cycle by the phage [would] be successful, producing viable phage progeny."

Answer: Fixed.

Page 27, Fig. 2: graph C: a unit such as (-fold) should be included on the Y axis; graph D: the labels "Phage" and "Non-lysogen" for rows on the gel should be switched; legend: add U, uninfected

Answer: We switched the labels in graph D (thank you for noticing!) and added an indication to the legend for U indicating uninfected cells. Instead of adding "(-fold)" to the Y axis of panel C, which we thought may be confusing, the legend text provides specific details describing the Y-axis: "Y axis represents relative phage concentrations normalized to the value at the beginning of the infection. "

Page 28, Fig. 3: graph C is unlabeled and a unit such as (-fold) should be included on the Y axis.

Answer: We added the label to graph C. To clarify the units of the Y axis, we modified the legend text and it now reads: "Y axis represents relative phage concentrations normalized to the value at the beginning of the infection, as measured by Illumina sequencing."

Page 29, Fig. 4, legend: "Curves depict culture dynamics of strains [lacking] BREX..."

Answer: Fixed.

Page 37, Fig. S2, legend: "Presented are dynamics for all tested phages except for those already presented in Figure [1]. Axes are as in Figure [1]."

Answer: Fixed.

Referee #3:

Goldfarb and Sberro et al. describe a novel super-family of Bacteriophage Exclusion (BREX) systems in bacteria and archaea. Although these innate defense systems also include a previously characterized anti-phage system from Streptomyces (PglZWXY; Chivenova 1982; Sumby & Smith 2002), the current study is a major extension that reveals a the broad distribution of BREX in about 10% of the available bacterial and archaeal genomes, and the existence of six BREX sub-types. More specifically, thorough experimental analysis is described of the 6-component system of Bacillus cereus, by its functional expression in B. subtilis. The cluster of 6 genes encode: a NusB-like RNA-binding protein (BrxA), an unknown small protein (BrxB), a P-loop ATPase (PglY/BrxC), a methyl transferease (PlgX), an alkaline phosphatase (PlgZ), and a Lon-like protease (BrxL). It is demonstrated that the heterologous BREX system provides B. subtilis with significant protection from a set of lytic and lysogenic phages. The fact that the system does not protect against all tested phages is explained by assuming the existence of counteracting viral anti-BREX systems. In a series of experiments, the authors gain some first insight in the BREX mechanism. Unlike earlier reports on the Streptomyces Pgl-system, the BREX system prevents the production of phage upon the first infection, and no integration of phages is observed. BRX is not an abortive infection system, and it does not block phage adsorption. However, the fact that phage DNA levels do not increase upon infection, indicate that phage replication is significantly inhibited by the BREX system. Sequence analysis revealed that in the presence of BREX, the host DNA (but not the phage DNA) was methylated at TAGGAG motifs. Unlike classical Restriction/Modification systems, the phage DNA is not being degraded. Based on the in silico and experimental analyses the authors draw some conclusions on the functionality of the system and its
components. This study provides a strong basis for future unravelling of the molecular mechanism of this intriguing defense system.

Major concern
1. The authors admit that many of the mechanistic details of the BREX system remain to be elucidated. Obviously, it is not difficult to propose some additional experiments. However, given the large set of data provided, it seems fair to wait for a follow-up study that addresses these matters. One easy experiment could be implemented. The authors should compare plasmid transformation efficiencies of the B.subtilis strain with/without BREX, using a Bacillus-E.coli shuttle vector (produced in E.coli). This will reveal whether BREX is a dedicated anti-virus system, or rather a general anti-invader system.

Answer: We thank the reviewer for suggesting this experiment, which is indeed an informative addition to the paper. We performed the suggested experiment, and show

the results in a new supplementary figure (Fig S4). The results of this experiment are now described in the following paragraph in the Results section: “Many defense systems, including restriction enzymes and CRISPR, can confer resistance against both invading phages and plasmids. To examine whether BREX can also block plasmids, we compared plasmid transformation efficiency between BREX-containing and control cells, using three different plasmids (two integrative and one episomal, low copy plasmid, with sizes ranging between 6.7-8.8kb). No considerable reduction in transformation efficiency was observed for the two integrative plasmids, whereas a mild effect (~1 order of magnitude) was observed for the episomal plasmid (Fig S4). These results show that plasmids can also be targeted, to a certain extent, by the BREX system. None of the plasmids, however, was blocked as efficiently as many of the phages we tested (>5 orders of magnitude). This may suggest that the plasmids we used do not contain strong targets for BREX, or that BREX specifically targets other characteristics of foreign DNA that are inherent to phage infection.”

2. (p.18, line 1) - It is concluded that the function of the protease in type-1 can be replaced by a helicase in other subtypes. As these are completely different enzymatic activities, with distinct targets (protein vs. nucleic acid), the authors should elaborate on this.

Answer: Following this comment, as well as a comment by referee #4, we removed the relevant Discussion text that implied the interchangeability of the protease and helicase functions.

3. The authors should indicate if TAGGAG motifs are randomly distributed, or if they mainly occur in 5'UTRs as part of RBS. If there is a bias, the authors should discuss the potential implications.

Answer: Following the referee’s comment we performed the suggested analysis and found that only 42% of the TAGGAG instances are found within a protein coding gene in the B. subtilis genome. Considering that >85% of the bacterial genome is occupied by protein coding genes, this represents a substantial tendency for this motif to be localized in intergenic regions upstream to protein coding genes. In accordance, we added the following text to the discussion section: “The resemblance of the methylated TAGGAG motif to the consensus ribosomal binding site (RBS) of Bacillus, AAAGGAGG, may lead to a hypothesis on a putative linkage between the BREX functionality and translation initiation. It remains to be seen, however, whether the same motif is being methylated in all instances of the BREX system, or whether each system carries a different motif specificity as in the case of R/M systems.”

Minor concern

4. (p.6, line 9) - "these phages" > change to: "the former 7 phages"

Answer: The text was changed to: “these seven phages”.

5. (p.14, line 10 / p.18, line 23) - "co-horizontally transformed" > change to: "co-transformed horizontally".

Answer: The text was changed to “horizontally transferred together”

Referee #4:

1. Why did the authors chose to change the description of the resistance system, when a perfectly good precedent exists i.e. Phage Growth limitation. Moreover the term exclusion implies that phage are excluded from the host cells and the evidence is clearly not supporting this.

Answer: Although the BREX system and the PGL system share some of their genes, we believe that throughout the manuscript we demonstrated many differences between these two systems, both in terms of the gene composition and in terms of the mode of operation. In the various places where the PGL system is discussed in the literature, the PGL phenotype is synonymous with a very special mode of operation, allowing a first, productive cycle of phage infection where the progeny phage can attack PGL-lacking cells. Through a series of experiments described in our manuscript, we decisively demonstrated that this is not the case for the BREX system. We believe that it would be confusing to refer to the BREX system as a PGL system. Moreover, we do not think that the term exclusion is misleading. The Miriam-Webster dictionary defines: “To exclude: to prevent (someone) from doing something…” which is consistent with the activity of the BREX system.

2. In many places in the ms the authors assume that the annotation is proof of activity. For example in the abstract lines 6 and 7 state that a lon-like protease, an alkaline phosphatase, a DNA methylase and an ATPase are involved in BREX but there is no validation for their biochemical activities or even whether the genes are required.

Answer: We thank the referee for pointing this out. We changed the text throughout the manuscript to address this issue. For example, in page 4 the text now reads: “…four additional genes that encode a putative protease, a protein with an ATPase domain, a predicted RNA-binding protein and a gene of unknown function...”, and so on.

3. The role of the pglX homologue in BREX is demonstrated but not conclusively as it was not complemented. Given the instability of pglX homologues, it would be sensible to complement this mutation. None of the other genes thought to be required for BREX were shown to be required and yet all of the classification of the BREX systems depends

on these homologues being active - in particular the pglZ homologues.

Answer: We agree that more experiments would be needed in order to decisively demonstrate the requirement and roles of each of the genes in the BREX system, but think that such additional studies are out of the scope of the current manuscript. We have demonstrated that the six BREX genes are co-transcribed as two polycistronic mRNAs (encompassing brxA-brxB-brxC-pglX and pglZ-brxL, Fig 1C), providing strong support that these genes belong to a single, coherent system. Moreover, the extremely strong tendency of the BREX system's components to appear together in the genome (e.g. Figure 1A), is highly indicative of these components participating in the same system (please also see our more detailed response to point #5 of this referee).

We respectfully think that although we have experimentally studied only a single instance of the BREX system (belonging to the most common subtype), the proposed classification of BREX subtypes is still biologically important and would be useful for future studies. In past cases where complex defense systems were discovered, system typing and classification were highly instructive even before the function of the system and its component was revealed. One such obvious example is the case of the CRISPR system, where a number of papers, based on bioinformatics analyses only, described the protein components of the CRISPR system and predicted subclassification of CRISPR types (e.g. Haft et al 2005), which guided the entire field for many years afterwards.

4. The assumption that phage that are resistant carry systems that evade BREX is wrong; these phages may simply not encode a target for BREX.

Answer: We thank the referee for raising this point. To clarify this possibility, we modified the text and it now reads: "Alternatively, the resistance of phage φ105 and its relatives to the BREX system could also stem from intrinsic differences in the infection cycle of these phages making them immune to BREX-mediated defense, or because they do not encode a target for the BREX activity."

5. The typing of BREX systems is likely to breakdown as more combinations of genes close to pglY, (the single common homologue) are revealed. I don't think that the classification proposed would therefore be useful or sustainable. Any typing systems should be based on mechanism. The classification is flawed anyway as we don't know which genes are required.

Answer: We agree with the referee that the typing of BREX systems may be refined in the future following sequencing of more genomes. However we respectfully disagree that the proposed classification is useless. As also indicated in our response to point #3 of this same referee, in past cases where complex defense systems were discovered, system typing and classification were highly instructive even before the function of the system and its component was revealed. One such obvious example is the case of the

CRISPR system, where a number of papers, based on bioinformatics analyses only, described the protein components of the CRISPR system and predicted subclassification of CRISPR types (e.g. Haft et al 2005), which guided the entire field for many years afterwards.

As to the suggestion of the referee that "the classification is flawed anyway as we don't know which genes are required": It is well established in the literature that complex systems, and particularly defense systems, have a strong tendency of being physically connected on microbial genomes, forming operons or larger gene clusters. This is true for restriction enzymes, in which the restriction endonuclease is almost always present, in the genome, next to a the methylase and the specificity subunits; for toxin/antitoxin systems, that almost invariably appear consecutively on a single operon; and obviously for CRISPR systems, in which all genes belonging to a single CRISPR system are frequently consecutively found on the genome. Based on the same principle, the extremely strong tendency of the BREX system's components to appear together in the genome (e.g. Figure 1A), is highly indicative of these components participating in the same system. Moreover, our results showing that the six BREX genes are co-transcribed as two polycistronic mRNAs (encompassing brxA-brxB-pglY-pglX and pglZ-brxL, Fig 1C) provide further strong support that these genes belong to a single, coherent system.

6. P3. Paragraph 2, line 12. Insert 'motif' after 'methyltransferase'.

Answer: The word 'motif' was added as suggested.

7. P6 paragraph 2 line 5. The growth kinetics are done under conditions in which the MOI is low so requiring many phage bursts before host lysis is observed. This should be made clear in the Results text.

Answer: We thank the reviewer for pointing this out. To avoid confusion, we changed the text so it now reads: "Growth curves of BREX-containing bacteria infected with these seven phages at a multiplicity of infection (MOI) of 10^{-3}-10^{-4} were similar to the uninfected bacteria…"

We note that the defensive properties of the BREX system are also observed at high MOIs for most of the phage tested. This can be seen in Table 1 and, for specific phages, in figure 2 and figure 3.

8. The growth of the host strain does not appear to be optimal; as the data is not presented as a log OD600 against time, we cannot see if the bacteria were growing exponentially in the initial growth phase and there is a clear reduction in the doubling time at OD600nm of about 0.3 which is very low for Bacillus subtilis growing in LB. The phage amplification is not presented in these growth curves and I think this would be useful to get a complete view of the phage-host dynamics.

Answer: We apologize for the confusion. As all OD600 measurements were taken in 96-well microplates, the measured OD600 values are not comparable to such measurements taken in 1cm-wide cuvettes, and we assume that this is the source for the confusion. According to the Beer-Lambert equation, the measured OD is proportional to *L*, the distance that the light travels through the liquid medium, which is smaller in the 96 microplate setup than in the 1cm cuvettes. Practically, if the referee wants to compare the microplate-measured values to values they are accustomed to when measuring in cuvettes, they should multiply the Y-axis value by about three times, yielding numbers which we believe are consistent with normal growth of *B. subtilis* in LB.

As detailed in the Methods section, the bacterial cultures were grown in flasks until early log phase, at which time the phage infections were set up in a 96-well plate and optical density measurements were taken every 13 minutes in a plate reader. The time = 0 shown on the graphs, is the time at which the bacterial cultures were first exposed to the phage. We hope that it is clear from the steep line seen on the graphs at times following the infection, that the cultures are in exponential growth phase.
Because the optical density values obtained in the experiments are less than 1, plotting these values on a log scale graph would give negative values, which would be confusing to the reader and are not a standard practice for presentation of bacterial OD growth curve measurements. Instead, in order to make the text clearer we now specifically indicate that the measurements were performed in 96 well plates. Accordingly, the Results text was modified to read: "The phage sensitivity of *B. subtilis* strains either containing or lacking the BREX system was evaluated using both optical density measurements in a 96-well plate format, and double agar overlay plaque assays." The figure legend was also altered to read: "(D-G) Culture dynamics of phage infected wild-type (black) versus BREX-containing (red) strains of *B. subtilis* BEST7003. Bacterial strains were exposed to phage at Time=0 (hr), and optical density measurements were read in a 96-well plate format. …."

The small volumes used in the 96-well plate format preclude us from assaying phage growth in these experiments. However, the required phage growth experiments were in fact performed as part of different experiments, and are presented in figure 2B-D.

9. P7 paragraph 2, 4,5 and 6 lines up. This calculation is not clear and need further explanation/data to validate.

Answer: We apologize for the lack of clarity of this part of the text. This text describes the results of a one-step phage growth curve assay, which is a standard technique in the field (Carlson 2005) that is described in detail in the Methods section (page 22). To clarify the nature of the experiment and its readout we modified the relevant paragraph in page 7 and it now reads:

"To gain further insight into the nature of the incomplete BREX defense against these phages, we performed a one-step phage growth curve assay (Carlson 2005) with SPO1. Briefly, this experiment involves mixing SPO1-infected cells with a SPO1-sensitive B. subtilis cells and plating them together using an agar overlay method. Phage bursts from successful infections are visualized as a single plaque on a lawn from the SPO1-sensitive B. subtilis strain, enabling an evaluation of the number of phages that have adsorbed and completed a successful infection cycle (Methods). Enumeration of plaques during the first 45 minutes of the time course infection indicated that the SPO1 phage was able to complete the lytic cycle only in 9% ± 4 of the initially infected cells (Fig 2B). A delay in the kinetics of the phage cycle was also observed, with phage bursts observed 75 minutes and 105 minutes following infection of BREX-lacking and BREX-containing cells, respectively (Fig 2B). Together, these results suggest that the BREX system provides significant, but not complete, protection from infection by phages SPO1 and SP82G."

10. P8&9. A more detailed analysis over the initial time-points of growth post-infection need to be shown to demonstrate that there is no stalling of host cell division/ DNA replication. Note that the OD600 can continue to increase as cells filament if DNA replication is blocked. The claim that this system is not an abortive infection needs to be validated by viable counts of infected cells in BREX-plus cells.

Answer: We agree that our results cannot determine whether there is transient stalling of host DNA replication, and that more experiments would be needed to make any such conclusion. However, in this experiment we did not seek to test for such transient effects, but to examine whether BREX is an abortive infection system. Abortive infection systems lead the cell to commit suicide once infected, and in this case the growth arrest effect should be permanent (and not transient over the very early time points of infection as suggested by the referee). Since we show (Fig 3A) that even at a high MOI (5 phages per bacteria) there is no visible decline or even growth inhibition in the culture, we believe that our conclusion, that BREX is not an Abi system, is justified.

11. P9. The use of Illumina reads to show the amplification of phage DNA compared to host DNA is not reliable as host DNA could be degraded. This might be already known for phi3T but it would not be known for phi3T infecting BREX plus cells. This experiment should be removed. The data using the southern blot is safer, but the readers need to see the amount of total DNA that was loaded onto the gel and blotted onto the membrane.

Answer: We confirmed by agarose gel electrophoresis that the host DNA is not degraded by phi3T in BREX- or BREX+ cells. We now include, as a supplementary figure Fig S3, the gel image showing no visible degradation of host DNA during the infection cycle, for both BREX-containing and BREX-lacking strains. To clarify this point, we modified the text so it now reads: "Since host DNA is not degraded following φ3T infection (Fig S3), by mapping sequenced reads to the reference *B. subtilis* and φ3T

genomes we could quantify the number of φ3T genome equivalents per infected cell at each time point." We also added the following sentence to the legend text of figure 4: "Each lane was loaded by 200 ng total DNA."

12. P11. Last line. Just because the so-called type I BREX systems are most common in the database, this does not mean it is most common in nature. Our view of what is common is skewed by what has been cultured and sequenced.

Answer: Following the referee's comment, we changed to the text to more accurately describe the BREX distribution, and it now reads: "The BREX system is by far the most common system in this superfamily amongst the genomes sequenced thus far (Fig 5B)."

13. P12. Paragraph 3. This is pure speculation with no evidence to support the claim that the Pgl system requires two additional genes. In fact the evidence from Sumby and Smith suggests otherwise. Linkage does not prove any functional connection.

Answer: We agree with the referee that linkage alone is not evidence for functional connection. To clarify that this is a speculation, the text was modified and now reads: "Given that both these genes appear in the same order in the type 6 BREX system (Fig 5A), one may speculate that these genes play an integral part of the type 2 BREX (Table S4)."

14. Pages 13 and 14 are descriptive only that lack any conclusions or biological insights.

Answer: We agree with the referee that pages 13 and 14 are descriptive, but we believe that these descriptions are important for understanding the biology of the system, as they depict the genomic structure and domain properties of the genes in each of the BREX subtypes that we have characterized.

15. P17. The authors show that replicating phages in a BREX plus strain are not methylated but how many target sequences do they have?

Answer: We thank the reviewer for raising this point. The genome of phi3T contains 43 TAGGAG motifs. The text was modified to include this information, and now reads: "However, none of the 43 TAGGAG motifs present on the phage genome was found to be methylated at any of the time points sampled during infection."

16. P18 lline 1&2. What kind of biochemical activity can be carried out by both a helicase and a protease? This is pure speculation. There is more speculation in the next paragraph.

Answer: Following this comment, as well as a comment by referee #3, we removed the relevant Discussion text that implied the interchangeability of the protease and helicase functions.

17. Figure 1G. The growth kinetics of the phi105 infection is odd as both cultures BREX plus and BREX minus start to lyse at 6 hours. The pfu/ml over this time course would be informative. In fact the phi105 infections look more like the SPO1 infection of the BREX minus culture and suggests that BREX does not confer resistance to phi105 after all. The EoP of the phi105 cultures do not show plaques, but perhaps they are there but very small.

Answer: We agree with the reviewer in their suggestion that BREX does not confer resistance to phi105. The growth curves of BREX- and BREX+ strain infected with phi105 look very similar. We hoped that this was clear from the text on page 6:

"In contrast to the protection from phage infection observed with the first seven phages tested, phage resistance was not observed upon infection with phage *ϕ105* and its close relatives, *rho10* and *rho14*. Similar kinetics of cell lysis were observed for strains either containing or lacking the BREX system (Fig 1G; Fig S2)."

This result is reiterated in Table 1, where it is shown that phage *ϕ105* infection is not blocked by BREX, and there is a similar efficiency of plating, with *ϕ105* plaques formed in the presence of BREX.

18. Figure 2B. The pfu/ml in the BREXplus culture with SPO1 decreases by about 1 log over 40 minutes an this is explained as adsorption, yet the pfu/ml in the BREX minus culture does not decrease - pfu/ml simply start to increase after about 30 minutes. Why this difference?

Answer: The reviewer is correct in pointing out that there is a large decrease in the readout of the phage concentration in the BREX+ cultures in the first 40 minutes following infection with SPO1. This occurs because SPO1 is efficiently adsorbed to the BREX+ cells, but only a small fraction of the infected population completes a successful round of phage infection. As part of the one-step phage growth curve experiment shown in Fig 2B, the infected cells are mixed with a phage-sensitive B. subtilis strain and plated together with the sensitive strain using an agar overlay method (Carlson 2005). The plaques are formed on the sensitive strain, and the number of plaques is the readout for successful infection events. Hence, phages that were successfully adsorbed at early time points and completed a successful cycle produce a single plaque in this assay, and this is reflected by stability of the curve for the BREX minus cells – each phage produces a single plaque. In contrast, in BREX-containing cells phages are successfully adsorbed at early time points but most of them fail to complete a successful cycle, resulting in the observed decline of the curve.

The M&M section contains a detailed description of the one-step phage growth curve protocol (page 22), which is a standard technique in the field. In addition, we modified the Results text in page 7 to better explain the assay, and also as a response to point #9 of the same referee. The text now reads:

"To gain further insight into the nature of the incomplete BREX defense against these phages, we performed a one-step phage growth curve assay (Carlson 2005) with SPO1. Briefly, this experiment involves mixing SPO1-infected cells with a SPO1-sensitive B. subtilis cells and plating them together using an agar overlay method. Phage bursts from successful infections are visualized as a single plaque on a lawn from the SPO1-sensitive B. subtilis strain, enabling an evaluation of the number of phages that have adsorbed and completed a successful infection cycle (Methods). Enumeration of plaques during the first 45 minutes of the time course infection indicated that the SPO1 phage was able to complete the lytic cycle only in 9% ± 4 of the initially infected cells (Fig 2B). A delay in the kinetics of the phage cycle was also observed, with phage bursts observed 75 minutes and 105 minutes following infection of BREX-lacking and BREX-containing cells, respectively (Fig 2B). Together, these results suggest that the BREX system provides significant, but not complete, protection from infection by phages SPO1 and SP82G."

2nd Editorial Decision 29 September 2014

Thank you for the submission of your revised manuscript to The EMBO Journal and thank you especially for your patience during the review process. We have now received the comments from two of the former referees that are now convinced that your manuscript should be published in The EMBO Journal. There is, however, just one minor technical issue that need to be dealt with before I can officially accept your paper.

I have noticed that you have included a rather small section called "Supplementary text" in the supplementary information file. For clarity and concision, we do not accept supplementary materials other than supporting figures and extra materials and methods, which otherwise would interfere with a fluid reading of the manuscript. I would therefore like to ask you to move this text into the main body or the article (or remove it if you consider it of relatively minor importance).

As you have probably seen already, every paper now includes a 'Synopsis', displayed on the html and freely accessible to all readers. The synopsis includes a 'model' figure as well as 2-5 one-short-sentence bullet points that summarize the article. I would appreciate if you could provide this figure and the bullet points.

I would also like to mention that we now encourage the publication of source data, particularly for electrophoretic gels and blots but also for numerical data in graphs, with the aim of making primary data more accessible and transparent to the reader. Although optional at the moment, would you be willing to provide a PDF file per figure that contains the unprocessed scans of all or key gels used in the figures and/or Excel files with the raw data used to build your graphs? The files should be labeled with the appropriate figure/panel number, and should have molecular weight markers in the case of blots; further annotation could be useful but is not essential. The files will be published online with the article as supplementary "Source Data" files directly accessible from the relevant figure.

Finally, in an effort to ensure good reporting standards and to improve the reproducibility of published results, our guidelines to authors have been made fully consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Accordingly, we now require the submission of a completed author checklist (attached), which covers in a systematic manner your practices regarding animal welfare, human subjects, data deposition, statistics and research ethics. It needs to be filled (not all fields may apply to your study in particular) and returned to the editorial office, either via the online submission system as a supplementary file when you upload the rest of the files, or by email (contact@embojournal.org).

If you have any questions regarding this initiative or any other part of the publication process, please let me know. Please check below for a link to upload the required files.

Thank you very much again for your patience and congratulations on a successful publication!

REFEREE REPORTS:

Referee #3:

All concern that I raised have been addressed satisfactory - i reccommend publishing this interesting paper !

Referee #4:

The findings reported in they manuscript will be of wide interest to microbiologists. The conclusions are well supported by the data.

2nd Revision - authors' response 04 November 2014

Attached please find the manuscript and supplementary materials edited as requested. We also added an author contribution paragraph and a conflict of interest paragraph after the acknowledments. There are additional small edits in the affiliations and in Table 2. All edits are in track changes.

Also attached is the author checklist.

We also found small gene naming inconsistencies in the supplementary tables and in Figure 5. I am sending you the revised Figure 5 as well as all the supplementary tables corrected.

